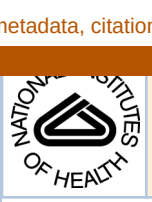

Published in final edited form as:
Mol Cancer Res. 2011 April ; 9(4): 430–439. doi:10.1158/1541-7786.MCR-10-0277.

Apoptosis is the essential tumor suppression function of p53 during mouse carcinoma progression

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Abstract

The high frequency of p53 mutation in human cancers indicates the important role of *p53* in suppressing tumorigenesis. It is well established that the *p53* regulates multiple, distinct, cellular functions such as cell cycle arrest and apoptosis. Despite intensive studies, little is known about which function is essential, or if multiple pathways are required, for p53-dependent tumor suppression *in vivo*. Using a mouse brain carcinoma model that shows high selective pressure for *p53* inactivation, we found that even partially abolishing p53-dependent apoptosis by *Bax* inactivation was sufficient to significantly reduce the selective pressure for p53 loss. This finding is consistent with previous reports that apoptosis is the primary p53 function selected against during *E*μ-myc induced mouse lymphoma progression. However, unlike observed in the *E*μ-myc induced lymphoma model, attenuation of apoptosis is not sufficient to phenocopy the aggressive tumor progression associated with complete loss of p53 activity. We conclude apoptosis is the primary tumor suppressive *p53* function and the ablation of additional *p53* pleiotropic effects further exacerbates tumor progression.

Keywords

Apoptosis; Tumor Protein p53; Selective Pressure; Brain Carcinoma; Tumor Progression

Introduction

p53 is frequently mutated in a wide array of human cancers (Hollstein et al., 1991; Petitjean et al., 2007). A variety of cell stresses, such as DNA damage, hypoxia, or cell-cycle aberrations, evoke a p53 transcriptional response, which modulates multiple physiological pathways, including cell-cycle arrest, apoptosis, genome surveillance, senescence, and angiogenesis (Dameron et al., 1994; Ko and Prives, 1996; Levine, 1997; Vogelstein et al., 2000; Vousden and Lu, 2002). The pleiotropic effects of *p53* make it difficult to distinguish whether a single p53-dependent function is uniquely responsible for the active selection against p53 activity during tumorigenesis, or if selective pressure is directed against the p53's aggregate activities, since loss of any one or combination of these functions could account for the prevalence of *p53* mutations among human cancers.

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The authors have no potential conflicts of interest.

Several investigators (Eischen et al., 2001; Hsu et al., 1995; Post et al., 2009; Schmitt et al., 2002; Sluss et al., 2010) have attempted to dissect the functions of *p53 in vivo* using the *Eμ-myc* transgenic mouse model of B-cell lymphoma (Harris et al., 1988) in which the immunoglobulin heavy chain enhancer drives expression of c-*myc*. In these lymphomas, apoptosis is the essential p53-dependent function suppressing tumor progression (Eischen et al., 2001; Schmitt et al., 2002). Reduced activity of *Bax*, a downstream effector of p53-mediated apoptosis, circumvented *p53* mutations usually acquired during progression (Eischen et al., 2001). Further evidence for the importance of apoptosis on the selective pressure against *p53* is the reduced frequency of *p53* loss of heterozygosity (LOH) in lymphomas that harbor a single *p53* null allele (*Eμ-myc*;$p53^{+/-}$) (Schmitt et al., 2002). Over-expressing either the *Bcl2* gene or a dominant-negative form of *caspase 9* blocks apoptosis and dramatically reduces *p53* LOH. Moreover, lymphomas in which apoptosis is completely abolished acquire an aggressive *Eμ-myc* phenotype, despite retention of the wild type *p53* allele, indicating reduced apoptosis not only reduces selective pressure against *p53*, but is also sufficient for lymphoma progression.

Whether apoptosis is the essential p53-dependent tumor suppressive function in epithelium-derived carcinomas has not been studied *in vivo*. Here we investigated the selective pressure against *p53* activity using a brain cancer model we previously described (Chen and Van Dyke, 1991). In this model, we directed expression of a truncated SV40 T antigen (T_{121}) in the mouse choroid plexus epithelium (CPE), and evaluated the effects of *p53* loss on tumor progression. The CPE lines the ventricles of the mammalian brain, forming the blood-brain barrier, and produces cerebral spinal fluid. Normally, CPE cells cease dividing within two weeks of birth. The T_{121} oncoprotein specifically inactivates pRb-related pocket proteins (pRb, p107, and p130), which causes cell-cycle re-entry. The unscheduled proliferation triggers apoptosis or predisposes cells to dysplastic growth followed by progression to carcinoma (Chen and Van Dyke, 1991; Symonds et al., 1994).

p53 plays a significant role in suppressing tumorigenesis in transgenic (Tg)T_{121} mice. Removing *p53* activity greatly reduces the apoptosis elicited by T_{121} expression in this brain tumor setting, with approximately half the apoptosis mediated by the *p53* effector gene, *Bax* (Symonds et al., 1994; Yin et al., 1997). *TgT_{121}* mice heterozygous or homozygous for a *p53* null allele develop CPE tumors with shorter latency than *TgT_{121}* mice with wild type *p53* alleles. There is immense selective pressure to inactivate the functional *p53* alleles in both *TgT_{121};$p53^{+/-}$* (100%) and *TgT_{121};$p53^{+/+}$* (38%) CPE tumors (Lu et al., 2001; Symonds et al., 1994). Furthermore, all tumors from *TgT_{121};$p53^{+/-}$* and *TgT_{121};$p53^{+/+}$* mice that inactivate *p53* were highly angiogenic, yet tumors lacking *p53* activity displayed little or no aneuploidy (Lu et al., 2001), contrary to the widely held view that *p53* is largely responsible for surveying genomic integrity.

The correlation between accelerated tumor progression and decreased apoptosis suggests a critical role for apoptosis in executing tumor suppression by *p53* in our brain tumor model. However, other p53-mediated functions, such as angiogenesis inhibition (Yin et al., unpublished results), or senescence may also subject the cell to selective pressure for p53 loss. In the present study, we explored the significance of p53-dependent apoptosis in CPE tumor suppression. We hypothesized lower apoptosis levels should decrease selective pressure for *p53* loss. We show reducing Bax-mediated apoptosis does indeed prevent the loss of *p53* activity; whereas, deficiency of an alternative p53-effector not involved in apoptosis, *p21*, does not alleviate selective pressure against *p53*. Loss of p53-dependent apoptosis, however, is not sufficient for progression to the more aggressive brain tumor phenotype associated with the complete absence of *p53* activity. The subsequent loss of pleiotropic *p53* functions allows tumors to acquire additional, distinct tumor phenotypes. While the influence of p53 effectors is likely cell type- and condition-specific, apoptosis

serves as the primary *p53* function that is actively selected against during tumor progression in both our brain carcinoma model and the *Eμ-myc* lymphoma model.

Materials and Methods

Mice

TgT_{121} transgenic mice (C57BL6/J; DBA2, F1 hybrid background) were previously described (Chen and Van Dyke, 1991; Saenz Robles et al., 1994; Symonds et al., 1994). $Bax^{-/-}$ mice (C3H), $p53^{-/-}$ (C57BL6/J) and $p21^{-/-}$ (C57BL6/129 hybrid) mice have been described (Brugarolas et al., 1995; Jacks et al., 1994; Knudson et al., 1995). $TgT_{121};Bax^{-/-}$ and $TgT_{121};Bax^{+/-}$ mice were generated by mating male TgT_{121} mice with female $Bax^{-/-}$ mice (Male $Bax^{-/-}$ mice are infertile). $TgT_{121};Bax^{+/-};p53^{+/-}$, and $TgT_{121};Bax^{-/-};p53^{+/-}$ mice were generated by mating $TgT_{121};Bax^{+/-}$ with $p53^{+/-}$ or $p53^{-/-}$ and interbreeding their progeny. $TgT_{121};Bax^{-/-}$ females were not used in breeding because they die around 2–3 months (Yin et al., 1997). $TgT_{121};p53^{+/-};p21^{+/-}$ mice and $TgT_{121};p53^{+/-};p21^{-/-}$ mice were produced by mating $TgT_{121};p53^{+/-}$ mice with $TgT_{121};p21^{-/-}$ mice and interbreeding their progeny.

Because the *Bax* null and TgT_{121} stock mice are maintained distinct background strains (C3H and c57BL6/DBA2 hybrid, respectively), we included in our analysis $TgT_{121};Bax^{+/+}$ sibling controls to avoid the potential pitfall of comparing tumors among mice of mixed genetic background strains.

Histology

Mouse brains were fixed in 10% formalin, embedded in paraffin, and cut in 5 0 μm sections. To examine tumor morphology, brains were sectioned for 6 to 10 consecutive layers with a 50 μm interval between each layer, and then 5 μm sections from each layer were stained with hematoxylin and eosin.

RNA *in situ* hybridization

Sections were treated and hybridized as previously described (Lu et al., 2001). The *p21* anti-sense probe was generated by T7 transcription of an EcoRI-linearized pBS-KSp21 template. The *Bax* anti-sense probe was generated by T3 transcription of a ApaI-linearized pBS-SKpBax template. Probes were labeled with [γ-^{35}S] UTP (5×10^4 cpm/μl) and hybridized to slides at 50°C overnight. Slides were exposed for 3 days (*p21*) or 8 weeks (*Bax*).

p53 immunohistochemistry

Paraffin embedded sections were boiled in Trilogy buffer (Cell Marker, Arkansas) for 15 min and then cooled at room temperature for 30 min. Sections were blocked in 10% normal goat serum (NGS) in phosphate buffered saline for 1 h at room temperature. Sections were incubated with rabbit anti-mouse p53 polyclonal antibody (Novocastra Laboratories Ltd., UK) at 1:600 dilution in 10% NGS at 4°C overnight. The secondary antibody staining and peroxidase enzymatic reaction were performed using an ELITE kit (Vector Laboratories Inc., California). After applying the DAB substrate, sections were incubated for 3–6 min and the staining was monitored by microscopy. Slides were counterstained with methyl green.

Statistical Analysis

Fisher's exact test was used to evaluate the significance of differences in p53 inactivation frequencies between tumors derived from alternative genotypes. p-values less than 0.05 were deemed significant.

Results

To assess whether apoptosis is the essential p53 tumor suppressor function selected against during brain tumor progression, we examined the frequency of *p53* inactivation in TgT_{121} mice with *Bax* deficiency. *Bax* mediates nearly half of apoptosis in this brain tumor model (Yin et al., 1997), although, *Bax* deficiency alone, without TgT_{121}, does not cause abnormal proliferation or tumorigenesis in CPE. We assessed *p53* activity two ways: first, we evaluated the histology of TgT_{121} CPE tumors as an indicator of the tumor's p53 status, as we have previously determined that p53 activity directly correlates with tumor histology in this model (Lu et al., 2001). Second, we assayed gene expression levels of the *p53* target gene, *p21* (el-Deiry et al., 1993). A functional assessment of p53 status is important since diverse mutations could render p53 inactive. Importantly, we have shown that *p21* transcription depends on p53 activity, and *p21* does not play a direct role in p53-dependent apoptosis in this brain carcinoma model (Lu et al., 2001).

TgT_{121} mice develop tumors with two distinct morphologies we designated Type I and Type II tumors (Table 1), which we previously demonstrated correspond to *p53* functional status (Lu et al., 2001): Type I tumors invariably show loss of p53 function (100%, n=34 tumors), while Type II tumors retain p53 function. Type I tumors have greater tumor volume and higher cell density, with abundant blood vessels and perivascular polarity, whereas Type II tumors show a dysplastic morphology and pleomorphic nuclei, with overall smaller tumor volume and fewer blood vessels. Type I tumors are more rare than Type II tumors in TgT_{121} mice (38% vs. 62%, respectively), presumably because the Type I phenotype requires loss of both active *p53* alleles.

To test the effect of reducing Bax-mediated apoptosis on brain tumor histology, we generated TgT_{121} mice that differed with respect to their *Bax* genotype ($^{+/+}$, $^{+/-}$, $^{-/-}$). We confirmed that the histological characteristics of tumors derived from TgT_{121},$Bax^{+/+}$ sibling mice were consistent with our previous observations of TgT_{121} mice, for both the Type I (Fig. 1 A, C) and Type II (Fig. 1 B, D) tumors, despite the mixed genetic backgrounds generated by intercrossing the two mouse strains. Importantly, the mixed genetic background in the present study had little effect on the tumor spectrum we previously reported. The frequency of Type I tumors in TgT_{121};$Bax^{+/+}$ mice is 35% (n=16), very similar to our previous observation in TgT_{121} mice (38%) (Lu et al., 2001). Using the tumor frequencies of TgT_{121};$Bax^{+/+}$ mice as a baseline for comparison, we scored the tumor morphologies among TgT_{121};$Bax^{+/-}$ and TgT_{121};$Bax^{-/-}$ mice. For both heterozygous and homozygous mice, *Bax* gene status significantly altered the spectrum of TgT_{121} tumors relative to *Bax* wild type mice ($p < 0.05$). Among TgT_{121};$Bax^{+/+}$ control mice (n=17) 35% of tumors were Type I. In contrast, among TgT_{121};$Bax^{+/-}$ mice (n=40) the percentage of Type I tumor was reduced to 12.5%, and among TgT_{121};$Bax^{-/-}$ mice (n=14), we observed no Type I tumors (Fig. 1 E). Consistent with our hypothesis, when apoptosis was reduced through lower *Bax* levels, fewer tumors displayed the Type I phenotype that is associated with complete loss of p53 function.

To confirm our histological assessment of *p53* activity, we assayed *p21* expression levels as a second indicator of *p53* function, since *p21* expression depends on p53 in this system (Lu et al., 2001; Pan et al., 1998). We measured *p21* expression using RNA *in situ* hybridization in tumors from TgT_{121};$Bax^{+/+}$ (n= 8 Type I and n=8 Type II), TgT_{121};$Bax^{+/-}$ (n= 4 Type I and n=2 Type II) and TgT_{121};$Bax^{-/-}$ (n= 4 Type I and n=2 Type II) mice. Consistent with the morphological assessments, all Type I tumors lost *p21* expression, while the Type II tumors maintained *p21* expression (Fig. 2). The *p21* sense probes did not show signal above background (data not shown). These results are summarized in Fig. 5A. Taken together, our analysis of tumor histology and *p21* gene expression indicate that although *Bax* mediates

only half of p53-dependent apoptosis in our brain tumor model, reduced *Bax* activity greatly reduces the frequency of *p53* inactivation.

Next, we assayed the frequency of p53 inactivation in tumors of mice that harbored a single engineered mutant *p53* allele. The rationale for these experiments was twofold: First, with only a single p53 allele, tumors would be more susceptible to complete loss of p53 function, thus making a more sensitive assessment of p53 activity levels. Indeed, 100% of tumors derived from $TgT_{121};p53^{+/-}$ mice progress to the Type I phenotype and virtually all (94%) lose the active p53 allele (Lu et al., 2001). Our second rationale was based on our observation that the majority of $TgT_{121};Bax^{-/-}$ mice (12 out of 14) developed severe hydrocephaly (Fig. 1F) which could also contribute to a shorter lifespan, in addition to CPE tumorigenesis. The median survival time of *Bax* null mice ($TgT_{121};Bax^{-/-}$) is considerably shorter than $TgT_{121};Bax^{+/+}$ mice (t_{50}= 12 vs. 31 weeks, Fig. 3A,B). The shortened timeframe among the $TgT_{121};Bax^{-/-}$ mice might be insufficient to allow tumor cells to lose both p53 alleles and display the Type I tumor phenotype; although, we know that Type I CPE tumors can develop in TgT_{121} mice as early as 8 to 9 weeks (Lu et al., 2001 and Lu unpublished data).

We generated both $TgT_{121};Bax^{+/-};p53^{+/-}$ and $TgT_{121};Bax^{-/-};p53^{+/-}$ mice and, as above, characterized *p53* inactivation by evaluating tumor histology (Fig. 4 A, C) and *p21* expression (Fig. 4 B, D). *Bax* heterozygosity ($TgT_{121};Bax^{+/-};p53^{+/-}$), only modestly reduced the incidence of Type I tumors we previously observed among mice wild type for *Bax* (i.e. $TgT_{121};p53^{+/-}$) (87% vs. 100%, respectively) (Fig. 3 C). In stark contrast, and consistent with our prediction, the incidence of Type I tumors (20%) was greatly reduced among *Bax* nullizygous mice ($TgT_{121};Bax^{-/-};p53^{+/-}$) (Fig. 3 C). Moreover, the lower frequencies of Type I tumors observed in animals with reduced *Bax* activity ($TgT_{121};Bax^{+/-};p53^{+/-}$ and $TgT_{121};Bax^{-/-};p53^{+/-}$) indicates a *bona fide* shift in tumor spectrum, and not an artifact caused by the co-morbidity of hydrocephaly, since $TgT_{121};p53^{+/-}$ mice develop Type I tumors with a latency (t_{50} = 9 weeks) within the expected lifespan of *Bax* null mice (Fig. 3 C,D and Lu et al., 2001). Furthermore, p21 expression directly correlates with differences in tumor morphology, as we have shown previously (Lu et al., 2001), and therefore serves as an indicator of p53 functional status. Taken together, these results are consistent with a model in which reduced *Bax*-mediated apoptosis alleviates selective pressure against *p53* even in the context of tumors sensitized to *p53* loss.

As an additional control for these experiments, we also assessed the effects of loss of *p21*, a *p53* downstream cell cycle arrest gene, on selective pressure against p53 activity. We reasoned if selective pressure is working primarily against p53 apoptotic activity, then the incidence of Type I tumors should remain high even when we mutate a p53-effector that is not involved in apoptosis. *p21* deficient mice are more prone to tumorigenesis in multiple tissues at old age (Martin-Caballero et al., 2001). However, *p21* does not contribute to *p53*-induced apoptosis in the TgT_{121} brain tumor model in the absence of DNA damage (Lu *et al.*, 2008). We generated a cohort of $TgT_{121};p21^{-/-};p53^{+/-}$ mice, then used p53 immunostaining to evaluate *p53* status in their tumors (Fig. 4 E–H). Among the tumors examined (n=17) 76% were Type I tumors, 18% were Type II tumors, and a single mouse developed both tumor types (Fig. 3 C). Since the Type I tumor frequency is significantly higher than we observed in $TgT_{121};Bax^{-/-};p53^{+/-}$ mice (82% vs. 20%, $p<0.05$), we conclude that selection against p53 remains high despite the absence of *p21* function. These results are consistent with a model where a p53-dependent function, which is not mediated by *p21*, is actively selected against during brain carcinoma progression. Furthermore, because *p21* deficiency also causes hydrocephaly to a similar extent as *Bax* deficiency in TgT_{121} and $TgT_{121};p53^{+/-}$ mice (data not shown), it also helps to rule out the possibility that

hydrocephaly directly affects the selective pressure against p53 during CPE tumor progression.

Given the importance of *Bax* in p53-dependent apoptosis in CPE, and our observation that *Bax* deficiency reduced the selective pressure against *p53* during CPE tumor progression, we reasoned that the *Bax* gene itself might also be subject to selective pressure. Among human cancers, reduced *Bax* expression is associated with poor clinical outcome in ovarian cancer (Tai et al., 1998), metastatic breast adenocarcinoma (Krajewski et al., 1995), and squamous cell carcinoma (Xie et al., 1999). *Bax* frameshift mutations have also been observed in colorectal cancers (Rampino et al., 1997). We tested this hypothesis using *in situ* hybridization to determine *Bax* gene status in $TgT_{121};Bax^{+/-}$ tumors (n=8). No tumors showed loss of *Bax* expression (Fig. 5). Negative control *Bax* sense probes did not show signal above background (data not shown). Although this assay cannot rule out *Bax* loss of function caused by frameshift or missense mutation, we suspect *Bax* is not subject to high selective pressure in this tumor setting. This result is perhaps not surprising, since *Bax* mediates only 50% of *p53*-dependent apoptosis in our brain tumor model, and losing additional pleiotropic p53 effects would confer greater advantage to tumors than *Bax* loss alone.

Discussion

The p53 tumor suppressor plays important role in cell cycle control, apoptosis, senescence, DNA repair, genomic stability maintenance, and regulation of angiogenesis. Together, these diverse functions contribute to p53's tumor suppressive activity. Until recently, little was known about which p53 function is essential for tumor suppression *in vivo*, and therefore actively selected against during tumor progression. During lymphomagenesis apoptosis appears to be the primary p53 function selected against (Schmitt et al., 2002), since selective pressure for *p53* loss was alleviated by reduced apoptosis levels (Eischen et al., 2001). Because the roles of p53 effectors are likely cell-and condition-specific, it is unclear whether apoptosis is also the essential target for selection during carcinoma progression.

To address this issue, we used the well-characterized brain carcinoma model in which CPE tumors are induced by expression of a T antigen mutant, T_{121}, transgene in choroid plexus epithelial cells (Symonds et al., 1994). An advantage of the TgT_{121} model is the immense selective pressure against p53 activity during CPE tumor progression. The frequencies of p53 inactivation during TgT_{121} or $TgT_{121};p53^{+/-}$ tumor progression were 38% and 100% respectively (Lu et al., 2001). To test the contribution of p53-dependent apoptosis to brain carcinoma suppression, we reduced p53-dependent apoptosis by inactivating a single downstream apoptotic gene, *Bax*. The absence of *Bax* activity ($TgT_{121};Bax^{-/-}$) completely suppressed the inactivation of p53, compared to the one-third of tumors derived from $TgT_{121};Bax^{+/+}$ mice that showed p53 inactivation. These results are consistent with our hypothesis that the reduced level of *Bax*-mediated apoptosis alleviates the selective pressure against p53.

A potential confounding factor in our experiments was the shortened lifespan of $TgT_{121};Bax^{-/-}$ mice compared to $TgT_{121};Bax^{+/+}$ mice (9 weeks vs. 32 weeks) that likely results from the combination of rapid tumor progression and high incidence of severe hydrocephaly. A shorter time frame might be insufficient to lose both *p53* alleles required to reveal the Type I phenotype. To avoid this potential problem, we analyzed tumor progression in mice harboring a single *p53* null allele ($TgT_{121};Bax^{+/-};p53^{+/-}$ and $TgT_{121};Bax^{-/-};p53^{+/-}$ mice), since $TgT_{121};p53^{+/-}$ mice develop Type I tumors (*p53* inactive) with much shorter latency than $TgT_{121};p53^{+/+}$ mice. Even in the sensitized background of p53 heterozygosity, the absence of *Bax* activity significantly reduced

selective pressure against *p53*. To further exclude the possibility that severe hydrocephaly directly affected selective pressure against *p53*, we also analyzed TgT_{121};$p21^{-/-}$; $p53^{+/-}$ mice, which have survival times similar to TgT_{121};$Bax^{-/-}$;$p53^{+/-}$ mice, and also develop severe hydrocephaly. The selective pressure against *p53*, evidenced by the high incidence of Type I tumors, was not diminished by the *p21* null background. We conclude a shorter lifespan associated with hydrocephaly does not preclude the Type I tumor phenotype. Instead, *Bax*-mediated apoptosis rather than *p21* function, is the target of selective pressure in tumors derived from brain epithelial cells.

Our studies also revealed notable distinctions between the brain carcinoma and B-cell lymphoma models. In the lymphoma model, *Bcl-2* over-expressing tumors advance to a phenotype that is morphologically indistinguishable from *p53*-deficient tumors, indicating that apoptosis alone is sufficient for tumor progression (Schmitt et al., 2002), even though others have shown non-apoptotic p53 functions also contribute to lymphoma tumor suppression (Liu et al., 2004; Ludwig et al., 1996; Post et al., 2009; Rowan et al., 1996). In contrast, in the brain carcinoma model, *p53* status corresponds to distinctive tumor biology and behavior. Tumors that completely lose p53 activity (Type I) show tumor phenotypes associated with more aggressive and malignant tumors; whereas, tumors that maintain *p53* activity (Type II), despite the absence *Bax* activity, show a comparatively less aggressive phenotype (Table 1). Therefore, p53-dependent pathways other than Bax-mediated apoptosis determine the advancement to malignancy unlike the lymphoma model. Other studies from our lab also point to the importance of losing p53 functions other than apoptosis during tumor progression. We have also characterized tumors derived from TgT_{121} mice harboring *p21* and/or *gadd45* null alleles. Only *p53* inactivation, but not loss of *Bax*, *p21*, *gadd45* (or *p21* and *gadd45* combined), leads to aggressive brain carcinoma (Type I tumors). p53 also appears to play a significant role in angiogenesis inhibition among CPE carcinomas, which we are currently characterizing (Yin *et al.* unpublished results). Taken together, these observations support our interpretation that the selective pressure for *p53* inactivation is due primarily to its role in apoptosis, while inactivating other *p53*-mediated functions contributes to further tumor progression.

Acknowledgments

We dedicate this manuscript to the memory of our colleague P. Anne Wolthuson who provided expert technical assistance to this project. The authors thank L. Lin and D. Moore at the UNC Lineberger Cancer Center for assistance with statistical analysis, R. Hill, and Stephen Jones for insightful discussion, the UNC Division of Laboratory Animal Medicine for animal husbandry, and the UNC Histopathology Core Facility for slide preparation. This work was supported by NIH grant 5-RO1CA46283 to TVD and ACS grant IRG-93-033-15 to KS.

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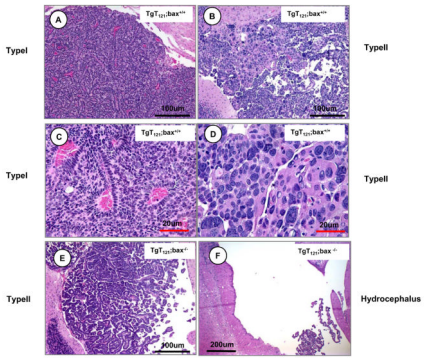

Figure 1. Choroid plexus (CP) tumor progression in *TgT*$_{121}$ mice with and without Bax gene deficiency

Two types of CP tumors develop in *TgT*$_{121}$ mice. Type I tumors are homogeneous tumors with larger overall tumor volume, greater cell density, with abundant blood vessels and peri-vascular CP cell polarity (A, C). Type II tumors show dysplastic cell morphology with pleomorphic nuclei, and lower nuclear:cytoplasmic ratio. Type II tumors also have relatively smaller overall volume, lower cell density, an little angiogenesis (B,D). *TgT*$_{121}$*;Bax*$^{-/-}$ mice develop Type II tumors (E) show and hydrocephaly (F). Images in panels C and D were captured at higher magnification.

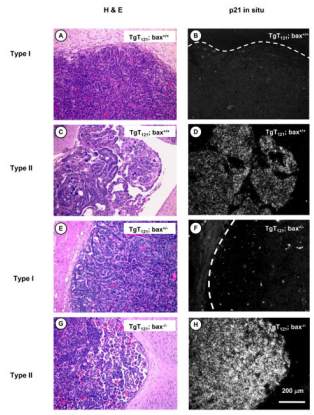

Figure 2. p53 status correlates with tumor histology

We used *p21* RNA *in situ* hybridization to determine p53 status in Type I tumors (A,B,E and F) and Type II tumors (C, D, G and H) derived from TgT_{121} mice with different *Bax* genotypes: $Bax^{+/+}$ (A–D), $Bax^{+/-}$ (E), and $Bax^{-/-}$ (H). *p21* RNA *in situ* hybridization was performed on tumor sections (n=8 Type I and n=8 Type II tumors) using an antisense *p21* riboprobe and viewed by dark-field microscopy (B,D,F and H). Adjacent sections were stained with H & E and viewed by bright-field microscopy to show the location and morphology of the CP (A,C,E and G). No *p21* expression is detected in Type I tumors (B and F), indicating p53 activity is lost in these tumors. In contrast, all Type II tumors maintain *p21* expression, indicating p53 is still functional. All pictures are equal magnification (bar=200 μm).

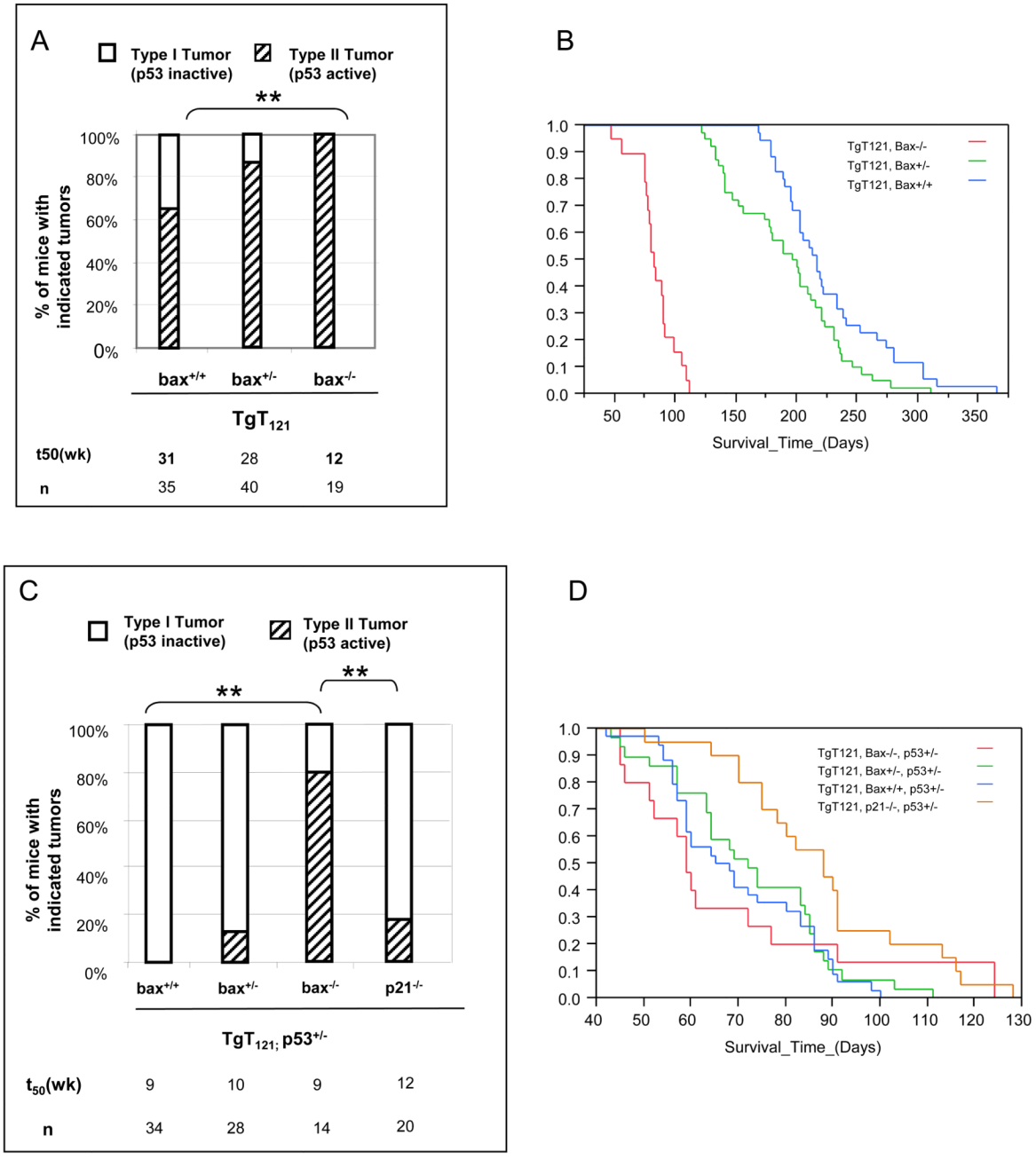

Figure 3. Bax deficiency decreases the frequency of p53 inactivation, associated with Type I tumor histology

(A) *Bax* deficiency reduces the frequency of Type I tumors. Some of the Type I and Type II tumors were analyzed by p21 RNA in situ hybridization to confirm p53 status as shown in Figure 2. (B) Surivival curves of mice with different genotypes as analyzed in (A). (C) *Bax* deficiency decreases selective pressure for p53 inactivation in TgT_{121} tumors that harbor a single *p53* allele. The vast majority of tumors derived from *Bax* null mice are Type II tumors (p53 active), indicating p53 loss is greatly decreased. In contrast, *p21* nullizygous mice, which show a similar lifespan and incidence of hydrocephaly as *Bax* nullizygous mice, still retain high p53 inactivation rates, indicating the effect is *Bax*-specific. Some of the tumors

were analyzed by p21 RNA in situ hybridization as shown in Figure 4. (D) Survival curves of mice with different genotypes analyzed in (C). (Fisher's exact test was used to compare tumor type distribution differences between different groups, ** indicates P<005).

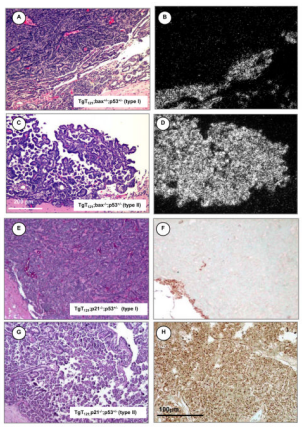

Figure 4. *p53* status corresponds to tumor histology

Type I (A,B and E,F) and Type II (C,D and G,H) tumors are derived from $TgT_{121};p53^{+/-}$ mice. H&E staining (A, C, E, G) were used to show the tumor morphology. *p21* RNA *in situ* hybridization (B, D) and immunohistochemistry (F, H) were used to examine p53's functional status.. In mice that harbor *Bax* mutant alleles *p21* RNA *in situ* hybridization reveals p53 function is lost in the Type I tumors (B), but not in the Type II tumors (D). Representative examples of Type I (n=8) and Type II (n=8) are shown. Among *p21* nullizygous mice (F,H), immunostaining also shows tumor histology corresponds to *p53* status (n=5 for each tumor type). Consistent with our previous results, all Type I but none of the Type II tumors show loss of p53 activity.. Panel A, B, E, F, G, and H are of lower magnification as indicated by the black bar in H. Panel C and D are of higher magnification as indicated by white bars.

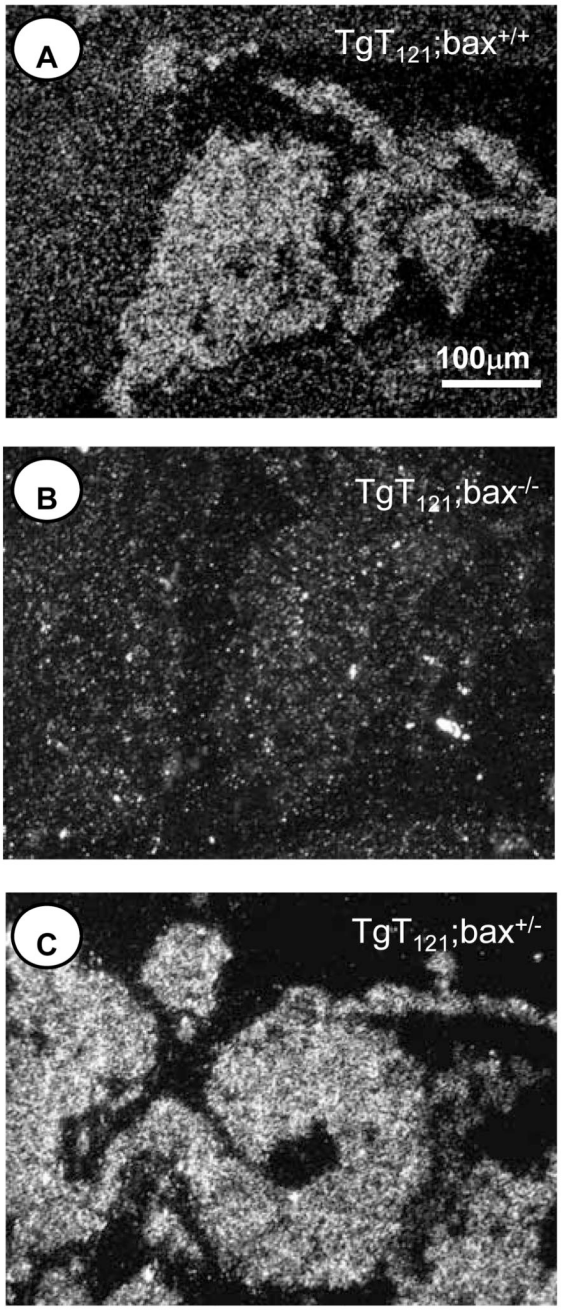

Figure 5. Loss of *Bax* expression is not detected in TgT_{121},$Bax^{+/-}$ terminal tumors

Given the importance of apoptosis, we reasoned there may also be selective pressure against a single active *Bax* allele. We analyzed *Bax* status in terminal stage *TgT12;*$Bax^{+/-}$ tumors (n=8) by RNA *in situ* hybridization using a *Bax* riboprobe. Panel C is representative of these results. Compared to Bax wild type (A) or nullizygous (B) controls, we see no evidence for loss of *Bax* transcripts in TgT_{121};$Bax^{+/-}$ tumors.

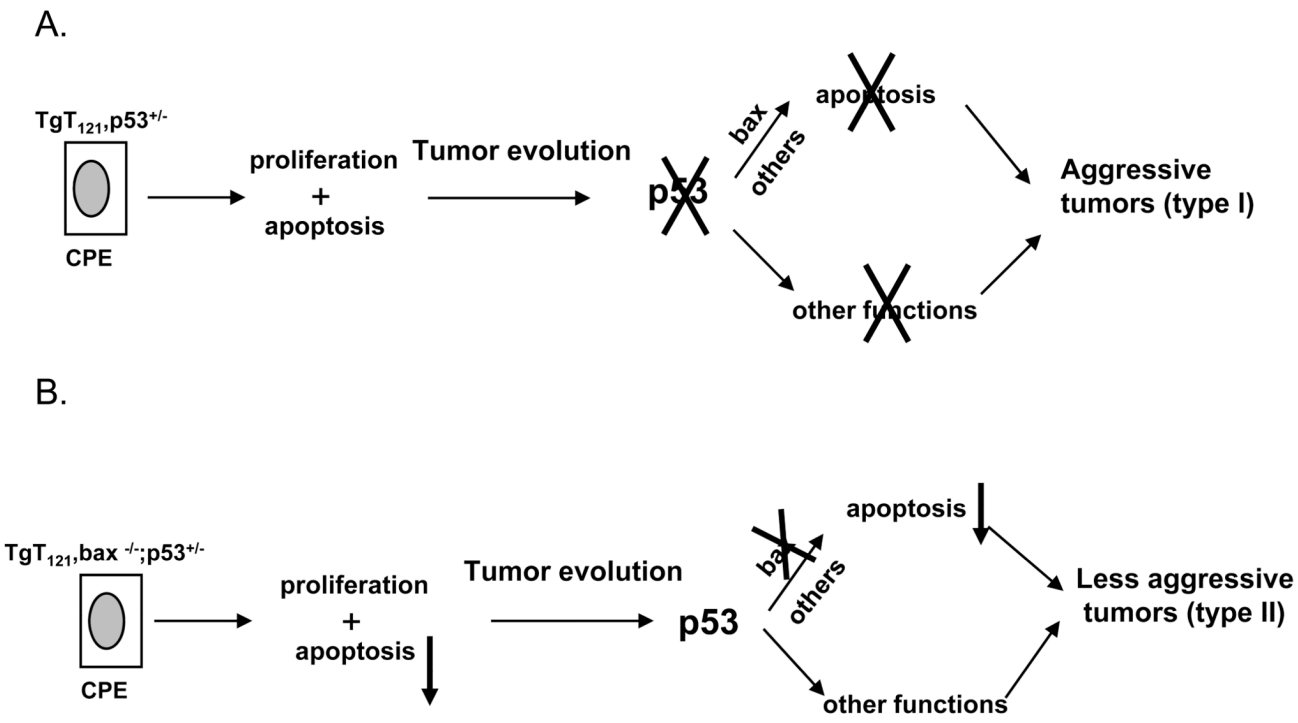

Figure 6. Two different tumor progression scenarios

(A) Among Type I tumors, the entirety of p53 functions are ablated by the spontaneous loss of *p53*, yielding aggressive, advanced tumors. When we reduce *Bax* activity (B), we decrease the selective pressure for *p53* loss. Thus, Type II tumors maintain the pleiotropic functions of *p53*, and develop tumors with distinct biology.

Table 1

Distinguishing features of Type I and Type II Choroid Plexus brain carcinomas.

	Type I	**Type II**
Gross Morphology	Greater tumor volume High cell density Homogeneous Cells Perivascular polarity	Smaller tumor volume Low cell density Dysplastic Cells No perivascular polarity
Tumor Blood Vessels	Abundant vessels	Fewer blood vessels
***p53* status**	Inactive	Active